Aus dem Diabetes Forschungsinstitut,
Lehrstuhl für Klinische Biochemie
(Diabetologie), Düsseldorf

Direktor: Professor Dr. Hans Reinauer

" Studies on proteolytic activities in heart muscle of diabetic rats "

Dissertation

zur Erlangung des Grades eines Doktors der Medizin
Der Medizinischen Fakultät der Universität Düsseldorf
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Summary

Induction of diabetes mellitus in rats ensued during the two weeks following injection of streptozotocin a loss in gain of heart weight, of protein and DNA content. During the same time interval total activity of acid proteinases (cathepsin D) was decreased by 9% versus a 19% increase in control hearts. The total activities of aminopeptidase, alkaline proteinase and free inhibitor increased to a lesser extent (6%, 80% and 17%) compared with controls (41%, 105% and 60%, respectively) over the two weeks period. The total aminopeptidase activity, alkaline proteinase activity and free proteinase inhibitor activity was consistently lower in diabetic hearts than in control hearts.

These data indicate that in the heart, in contrast to skeletal muscle, induction of diabetes mellitus is not followed by an increase of alkaline proteinase and aminopeptidase activity, suggesting a different response of these tissues to insulin deficiency.

Key words

Rat heart. Proteinases. Proteinase inhibitor. Diabetes mellitus.

Introduction

The regulatory effect of insulin on protein metabolism in muscle tissue has been found to be primarily a stimulatory one on protein synthesis [1,2]. Furthermore, there is evidence that insulin depresses protein degradation in skeletal muscle [3,4,5,6], although the in vivo relevance of this phenomenon has been questioned [7].

In diabetes mellitus, due to insulin deficiency, the anabolic effects of this hormone on muscle protein metabolism are absent, leading to a reduction of protein synthesis and an increase of protein breakdown [8].

A similar situation has been reported for heart muscle in the diabetic rat, in that protein synthesis is reduced [9,10] and protein degradation is enhanced [10].

In earlier studies we and others have shown that in the rat the activity of the skeletal muscle alkaline proteolytic system shows adaptive behaviour to alterations of insulin levels [11,12]. Since the change in alkaline proteinase activity closely correlates with changes in muscle protein metabolism, it appears that this proteolytic system is involved in enhanced muscle protein breakdown as has been observed in the diabetic rat [13]. This view is supported by results of Griffin and Wildenthal [14] as well as Mayer et al. [15] who found after induction of diabetes mellitus in rats an increase in activity of heart muscle alkaline proteinase(s). In both

investigations, however, the proteolytic activity was determined by measuring the autolysis of endogenous proteins, a method which does not allow to differentiate between an increase in proteolytic capacity and an enhanced substrate susceptibility to proteinases.

The present study was undertaken to assess the activity of the alkaline proteolytic system in rat heart when measured with the exogenous substrate, [^{14}C]haemoglobin, and to determine whether this activity shows adaptive behaviour to experimentally induced diabetes mellitus similar to that descibed by Griffin and Wildenthal [14] and Mayer et al. [15]. In addition, the overall activity of acid proteinases and aminopeptidases as well as the amount of free proteinase inhibitor [16] were determined during the two weeks after onset of streptozotocin-induced diabetes mellitus.

Materials and Methods

Chemicals

[^{14}C] haemoglobin was prepared as described by Roth et al. [17] using bovine haemoglobin (Sigma, München, F.R.G.) and K^{14}CNO (specific activity 50-60 mCi/mmol) from Amersham Buchler, Braunschweig, F.R.G.. Azocasein, α-chymotrypsin [E.C.3.4.21.1.] and deoxyribonucleic acid from calf thymus were from Serva, Heidelberg, F.R.G.. Leucine-4-nitroanilide and 3,4-diaminobenzoic acid were products of Merck, Darmstadt, F.R.G.. Streptozotocin was obtained from Boehringer, Mannheim, F.R.G., and LAB TROL was from Dade, Miami, U.S.A..

Experimental animals

40 male rats (Bor: WISW from Winkelmann, Borchen, F.R.G.) with a body weight of 170-190 g were divided into two groups. Diabetic rats: 25 rats were injected intravenously with 70 mg streptozotocin/kg body weight after a 24 h fast. Streptozotocin was dissolved in 1 ml of 0.1 M phosphate/citrate buffer, pH 4.5.

Control rats: After a 24 h fast 15 rats received an intravenous injection of 1 ml 0.1 M phosphate/citrate buffer, pH 4.5 After the injections all animals got food and water ad libitum.

2,4,6,8 and 14 days following the injections, 3 animals of the control group and five animals of the diabetic group, respectively, were sacrificed. The chest was opened, the heart was rapidly excised and washed in ice-cold saline. After weighing, the hearts were frozen in liquid nitrogen

and subsequently pulverized in a mortar to ensure complete homogeneity.

Preparation of heart muscle homogenate for measurement of acid and alkaline proteinase activity

0.2 g powered heart tissue was suspended in 2 ml of 2 mM Tris/HCl/1 M KCl/0.2% Triton X-100, pH 7.4, and homogenized in a MSE homogenizer for 1 min at 14000 rpm. The mixture was centrifuged for 10 min at 3000 x g (4°C) and the supernatant decanted and stored on ice. The precipitate was resuspended in 1 ml buffer, homogenized and centrifuged as described above. The supernatant fractions from both centrifugation steps were then combined and filtered through nylon mesh (100 µm). The clear filtrate was diluted with buffer consisting of 2 mM Tris/HCl/1 M KCl/0.2% Triton X-100, pH 7.4, to give a protein concentration of 7 mg/ml. This muscle crude extract was then divided into two aliquots, each of which was further diluted to arrive at a final protein concentration of 3 mg/ml. For measurement of the alkaline proteinase activity, the first aliquot was diluted with 250 mM Tris/HCl/1 M KCl, pH 9.0. To determine proteolytic activity at acid pH, the second aliquot was diluted with 100 mM sodium acetate/1 M KCl, pH 4.0.

Measurement of proteinase activity

Proteolytic activity was determined by incubating 0,1 ml of an aqueous solution of [^{14}C] haemoglobin (6%, w/v) with 0.2 ml muscle extract (3 mg protein/ml in pH 9 or pH 4 buffer) and 0.2 ml 250 mM Tris/HCl/1 M KCl/0.2% Triton X-100, pH 9.0, or 0.2 ml sodium acetate buffer, pH 4.0, for 60 min at 37°C. The reaction was stopped by addition of 0.1 ml 50% (w/v) trichloroacetic acid. Undigested protein was spun down at 11000 x g

for 5 min and the supernatant was measured in a liquid scintillation spectrophotometer. Blanks were stopped by addition of trichloroacetic acid prior to incubation at 37°C.
At each pH measured, the increase of trichloroacetic acid soluble radioactive peptides was linear with muscle protein concentrations ranging from 0.5 to 5 mg/ml and linear with time up to 3 hrs. Proteolytic activity is given as the amount of radioactive peptides (dpm) released into the trichloroacetic acid soluble fraction after an incubation for 60 min at 37°C.

Preparation of heart muscle homogenates for determination of aminopeptidase activity, DNA content and proteinase inhibitor activity

The procedure followed for preparing muscle crude extract (protein concentration: 7 mg/ml) was the same as described above, except that Triton X-100 was omitted from the homogenization buffer since this detergent was found to strongly inhibit (to about 70%) aminopeptidase activity.

Measurement of aminopeptidase activity

For measurement of overall aminopeptidase activity the (Triton-free) muscle crude extract was further diluted with 50 mM Tris/HCl/1 M KCl, pH 7.0, to obtain a protein concentration of 3 mg/ml. The activity was determined by using leucine-4-nitroanilide as substrate as described elsewhere [13]. In preliminary experiments hydrolysis of leucine-4-nitroanilide had been found to be optimal between pH 6.5 and 7.5, and a linear relationship for substrate hydrolysis was obtained between 1 to 6 mg/ml heart muscle protein and incubations lasting from 15 to 180 min.

Determination of proteinase inhibitor activity

Triton-free muscle crude extract was diluted with 2 mM Tris/HCl/1 M KCl, pH 7.4, to obtain protein concentrations of 1.5, 1.0 and 0.5 mg/ml, respectively. 0.05 ml of a solution of chymotrypsin (2.2×10^{-4} M in 2 mM Tris/HCl/1 M KCl, pH 7.4) was preincubated for 10 min at 21°C with 0.15 ml of differently diluted muscle extracts. The remaining chymotryptic activity was measured by adding 0.05 ml 3% (w/v) azocasein dissolved in 50 mM Tris/HCl, pH 8.5. This reaction mixture was incubated for 30 min at 37°C and stopped by addition of 0.5 ml 8% (w/v) trichloroacetic acid. After centrifugation (11000 x g, 5 min) the absorbance of the acid soluble material was measured at 366 nm. Values were corrected for caseinolytic activity present in the muscle homogenate and blanks. Chymotryptic activity measured in the presence of buffer instead of muscle extract was taken as 100% activity. The amount of chymotrypsin which under these conditions causes a change in absorbance of 0.001/min is defined as 1 unit (1 U). The slope of the titration curve for chymotrypsin, obtained from inhibition experiments with three different concentrations of muscle protein, was used to calculate the amount of muscle protein required to inhibit 1 U of chymotrypsin.

Measurement of DNA content

The DNA content was determined with 3,5-diaminobenzoic acid after diluting the Triton-free muscle crude extract with 2 mM Tris/HCl/1 M KCl, pH 7.4, to obtain a protein concentration of 5 mg/ml. The fluorescence test was performed as described by Afting and Elce [18] but omitting the pronase digestion step.

Protein concentrations were measured by the biuret method with LAB TROL as a standard.

Statistical analysis

Statistical significance of differences between mean values of data obtained with control animals and those of diabetic animals was determined by Student's t-test.

Results

Total body weight, heart weight, protein and DNA content

Successful induction of diabetes mellitus in rats of group D is demonstrated by a four-fold increased plasma glucose level, by strong glucosuria and by mild ketonuria (Table I).

The body weight of the rats in the control group increased by about 70 g during the two weeks of the experiment, while the body weight of diabetic rats increased by 12 g only (Table II). Similarly, the heart weight in normal rats showed a substantial increase (about 40%), whereas the heart weight of diabetic rats did not change significantly. Thus, while the heart weight/body weight ratio decreased in group D, it was unchanged in group C (Table II).

Growing of the hearts in normal animals, as illustrated by their weight increase and shown in Table II, was paralleled by a protein content, which was 34% higher two weeks after the beginning of the experiment (Table III). In contrast, no protein accumulation occurred in hearts of diabetic rats during the experimental period.

Since the DNA content in hearts of both groups of animals was statistically not different and since this content remained nearly unchanged during the experimental period, the

TABLE I PLASMA GLUCOSE LEVELS, GLUCOSURIA AND KETONURIA IN NORMAL AND DIABETIC RATS.

Blood glucose levels as measured at the days indicated in the first column are given as means ± S.E.M. for 3 control animals (group C) and 5 diabetic animals (group D). Glucosuria and ketonuria were determined with Glucetur-test strips (Boehringer,Mannheim, G.F.R.):(-) no, (+) mild, (+++) strong glucosuria or ketonuria.

Day	Group C			Group D		
	Blood glucose (mg/dl)	Glucosuria	Ketonuria	Blood glucose (mg/dl)	Glucosuria	Ketonuria
2	109 ± 1	-	-	435 ± 16	+++	+
4	111 ± 3	-	-	437 ± 12	+++	-
6	108 ± 3	-	-	432 ± 7	+++	-
8	116 ± 2	-	-	423 ± 20	+++	-
14	102 ± 3	-	-	466 ± 34	+++	-

TABLE II BODY WEIGHT AND HEART WEIGHT OF NORMAL AND DIABETIC RATS

Rats of 170–190 g body weight were divided into two groups: animals of group C were used as controls and animals of group D were injected with streptozotocin to induce diabetes mellitus. Afterwards, at the days indicated in the first column, body weight and heart weight of the animals were measured and are given together with the heart weight/ body weight ratio as means ± S.D. Values indicated with an asterisk are of statistically significant difference ($p < 0.05$) from the C group values.

Day	Body weight (g)		Heart weight (mg)		Heart wt./ Body wt. (mg/g)	
	C	D	C	D	C	D
2	182 ± 7	169 ± 8	620 ± 80	560 ± 50	3,40 ± 0,30	3.31 ± 0.32
4	201 ± 11	167 ± 10*	680 ± 50	530 ± 30*	3,38 ± 0.18	3.17 ± 0.07
6	198 ± 8	168 ± 19*	670 ± 30	530 ± 40*	3.38 ± 0.06	3.15 ± 0.21
8	223 ± 13	182 ± 12*	750 ± 80	570 ± 40	3.36 ± 0.18	3.13 ± 0.15
14	251 ± 17	181 ± 20*	860 ± 20	530 ± 40*	3.43 ± 0.19	2.93 ± 0.22*

TABLE III PROTEIN AND DNA CONTENT IN HEARTS OF NORMAL AND DIABETIC RATS.

Protein and DNA content as well as protein/DNA ratio in hearts of normal (C) and diabetic (D) rats were measured at the days after starting the experiment as indicated in the first column. Means ± S.E.M are given. Values indicated with an asterisk are of statistically significant difference ($p<0.05$) from the group values.

Day	Protein content (mg/heart)		DNA content (μg/heart)		Protein/DNA (mg/μg)	
	C	D	C	D	C	D
2	117 ± 7	92 ± 5*	730 ± 49	747 ± 29	0.160 ± 0.021	0.122 ± 0.003
4	132 ± 12	88 ± 3	981 ± 28	739 ± 29*	0.133 ± 0.008	0.119 ± 0.006
6	125 ± 8	90 ± 6*	808 ± 41	782 ± 58	0.154 ± 0.011	0.115 ± 0.007
8	152 ± 26	93 ± 5	833 ± 20	816 ± 38	0.151 ± 0.005	0.114 ± 0.006*
14	157 ± 11	90 ± 5*	895 ± 12	758 ± 66	0.175 ± 0.010	0.120 ± 0.005*

protein/DNA ratio was lower in diabetic rats when compared to that in the control group (Table III).

Aminopeptidase activity

The overall aminopeptidase activity in hearts of diabetic rats was significantly lower than in those of healthy animals and the activity increase of about 40%, observed in the course of two weeks in groups C was reduced to about 10% in group D (Table IV). As the protein content of the hearts was similarly increased, the activity expressed per mg protein remained unchanged and was not different either, when rats of group C and D were compared. The activity expressed per µg DNA was generally higher in normal rats because of the equal DNA content in hearts of both groups and the higher total activity in normal rats (Table IV).

Acid proteinase activity

As shown in Table V, the total proteolytic activity measured at pH 4 is lower in diabetic hearts as compared to normal hearts and, from 192000 dpm/heart at day 2 slightly decreased to 175000 dpm/heart at day 14, but the specific activity (activity/mg protein) was lower by about 18%. Since total proteolytic activity in normal hearts did not significantly change, the specific activity expressed per mg protein decreased due to an increase in protein content. When referred to the DNA content of the hearts, the acid proteinase activity was the same in both groups (Table V).

Alkaline proteinase activity

The data of Table VI clearly show that alkaline proteinase activity is lower in hearts of diabetic rats as compared to healthy control rats irrespective of the reference value

TABLE IV OVERALL AMINOPEPTIDASE ACTIVITY IN HEART MUSCLE

At the days given in the first column the aminopeptidase activity in hearts of normal (C) and diabetic (D) rats was measured with leucine-4-nitroanilid as substrate. Means ± S.E.M. are given. Values indicated with an asterisk are of statistically significant difference ($p < 0.05$) from the C values.

Day	Total activity (nMol/min x heart)		Activity/Protein (nMol/min x mg)		Activity/DNA (nMol/min x µg)	
	C	D	C	D	C	D
2	137.8 ± 1.6	103.6 ± 1.2*	1.18 ± 0.09	1.13 ± 0.07	0.189 ± 0.011	0.139 ± 0.005*
4	157.2 ± 7.5	105.6 ± 2.3*	1.20 ± 0.10	1.20 ± 0.05	0.160 ± 0.006	0.144 ± 0.007
6	143.6 ± 9.2	102.4 ± 5.7*	1.15 ± 0.09	1.14 ± 0.04	0.177 ± 0.003	0.132 ± 0.009*
8	180.5 ± 26.7	116.7 ± 6.2	1.20 ± 0.13	1.25 ± 0.05	0.189 ± 0.020	0.142 ± 0.004
14	194.8 ± 8.7	110.4 ± 5.7*	1.24 ± 0.05	1.23 ± 0.05	0.217 ± 0.007	0.148 ± 0.010*

TABLE V ACID PROTEOLYTIC ACTIVITY IN HEART MUSCLE

Proteolytic activity was determined at pH 4.0 with [^{14}C] haemoglobin as substrate. The activities in hearts of control (C) and diabetic (D) animals at day 2 to 14 after starting the experiment are given as means ± S.E.M.. The value indicated with an asterisk is of statistically significant difference ($p < 0.05$) from the C group value.

Day	Total activity (dpm x 10^{-3}/heart)		Activity/Protein (dpm/mg)		Activity/DNA (dpm/μg)	
	C	D	C	D	C	D
2	273 ± 49	192 ± 16	1878 ± 144	1828 ± 75	444 ± 41	256 ± 14*
4	232 ± 39	190 ± 24	1650 ± 152	1793 ± 190	235 ± 36	260 ± 36
6	272 ± 27	183 ± 31	1656 ± 176	1557 ± 216	340 ± 39	238 ± 46
8	268 ± 54	176 ± 22	1664 ± 223	1515 ± 130	273 ± 22	221 ± 36
14	327 ± 62	175 ± 15	1479 ± 132	1517 ± 121	364 ± 66	236 ± 24

chosen to compare the activity activity per heart, per protein or per DNA).

In both groups an increase of activity was observed throughout the experimental period (105% in group C and 82% in group D, when comparing the activities on day 2 and day 14). Since the heart protein content measured in normal animals increased in the same fashion, the specific activity of alkaline proteinase (dpm/mg protein) remained unchanged in this group of animals. The slight increase of specific activity observed in group D animals was statistically not significant. When expressed per µg DNA, the increase of activity was not significant, either (Table VI).

Proteinase inhibitor activity

The total inhibitory capacity was higher by about 60% and by about 17% in hearts from control and diabetic animals, respectively, when day 2 and 14 values are compared (Table VII). Hence, the inhibitory capacity measured in hearts of normal rats was higher than that in hearts of diabetic rats.

On the other hand, the inhibitory capacity expressed per mg protein was always higher in diabetic hearts than in control hearts. However, the increase of capacity as well as the differences between group C and D were statistically not significant at any day during the experimental period (Table VII).

Discussion

The present study clearly shows that during a period of two weeks following the induction of diabetes mellitus by injection of streptozotocin the gain in body weight is signi-

TABLE VI ALKALINE PROTEOLYTIC ACTIVITY IN HEART MUSCLE

Proteolytic activity was measured in heart muscle at pH 9.0 with [^{14}C] haemoglobin as substrate. The activities (means ± S.E.M.) in control (C) and diabetic (D) rats are given and the values indicated with an asterisk are of statistically significant difference ($p<0.05$) from the C group values.

Day	Total activity (dpm x 10^{-3}/heart)		Activity/Protein (dpm/mg)		Activity/DNA (dpm/μg)	
	C	D	C	D	C	D
2	53 ± 10	29 ± 5	374 ± 59	272 ± 43	81 ± 13	38 ± 6
4	81 ± 21	35 ± 6	562 ± 97	337 ± 60	82 ± 20	49 ± 10
6	88 ± 3	44 ± 9*	537 ± 45	368 ± 47*	109 ± 5	55 ± 7*
8	98 ± 25	42 ± 8	602 ± 112	355 ± 62	99 ± 15	54 ± 14
14	109 ± 15	53 ± 13*	501 ± 35	451 ± 105	121 ± 17	67 ± 14

TABLE VII INHIBITOR ACTIVITY IN HEARTS OF NORMAL AND DIABETIC RATS

The free inhibitor activity in hearts of normal (C) and diabetic (D) rats was titrated with bovine chymotrypsin at the days 2 to 14 after starting the experiment. Means ± S.E.M are given.

Day	Total activity ($IU \times 10^{-3}$/heart)		Activity/Protein (IU/mg)	
	C	D	C	D
2	489 ± 104	497 ± 28	4129 ± 626	5489 ± 573
4	646 ± 86	514 ± 39	5068 ± 1177	5909 ± 635
6	638 ± 110	530 ± 29	5170 ± 1049	6064 ± 680
8	701 ± 82	570 ± 64	4813 ± 908	6122 ± 758
14	786 ± 115	584 ± 82	5115 ± 1086	5751 ± 506

ficantly reduced and heart growth is almost completely abolished. In parallel, the protein content in the diabetic hearts remains constant. Different from this, protein content in normal hearts steadily increases.

According to the findings of several investigators [9,10] the stagnation of protein accumulation in diabetes is partly due to the inhibition of cardiac protein synthesis but is also due to an increase in protein breakdown.

In perfusion experiments with rat hearts Rannels et al. [19] found that insulin has no effect on total activity of cathepsin D. In agreement with these results our data show that total acid proteinase activity (which under the experimental conditions is mainly due to cathepsin D activity) is not significantly affected by insulin deficiency. At present, it is unclear, whether the fragility of lysosomes observed in the heart of diabetic rats [19] is related on increased protein breakdown. Although an in vitro degradation of cardiac myosin and actin by cathepsin D isolated from homologous tissue has been demonstrated [20], the lysosomal system appears not to be responsible for the in vivo degradation of cardiac myosin. Thus, the inhibitory effect of insulin on this degradatory process cannot be mimicked by chloroquine or leupeptine, although these substances, by affecting the pH in lysosomes and lysosomal cathepsins, respectively, reduce total protein degradation in heart [21].

On the other hand, Griffin and Wildenthal [14] as well as Mayer et al. [15] have measured an increased alkaline proteinase activity in heart muscle when comparing streptozotocin diabetic rats with normal rats. In contrast to these fin-

dings, our own results clearly show that throughout the experimental period the alkaline proteolytic activity is lower in diabetic hearts than in hearts of control animals. A possible explanation of this discrepancy could be that accelerated autolytic activity as determined by these authors is not due to a genuine increase of proteolytic capacity, but is due rather to increased susceptibility of muscle proteins to proteolytic action. However, with haemoglobin beeing used as a substrate in our experiments, this question cannot at present be answered. Another possibility could be that the proteolytic activity which is increased in the diabetic state [14, 15] with degrading muscle protein does not split haemoglobin. However, this would be contrary to the finding that alkaline proteinases present in skeletal muscle degrade both endogenous muscle proteins and radioactive haemoglobin [12, 13, 22] . In support of this notion a 'myosin cleaving protease' in heart muscle, active at alkaline pH [23], has been shown to degrade the exogenous substrate casein as well as myofibrillar proteins [24] and this enzyme is most likely to also split haemoglobin.

In an attempt to determine whether a lowered alkaline proteinase activity in diabetic hearts may be the result of an increase in proteinase inhibitor [16], free inhibitor was titrated with bovine chymotrypsin. It was found that during the 14 days of the experiment, the total amount of free inhibitor in diabetic cardiac muscle increased more slowly (+ 17%) than in normal hearts (+60%). These data suggest that in the insulin deficient state synthesis of the inhibitor is slowed down together with that of other proteins or, alternatively,

that more proteinase-proteinase inhibitor complexes are formed, resulting in a lower number of titratable inhibitor molecules and lower alkaline proteinase activity.

Irrespective of the underlying mechanism, our data show that alkaline proteinase activity is not increased in diabetic hearts as opposed to skeletal muscle alkaline proteinase activity [11,12,13] and therefore this enzyme system is unlikely to be involved in an accelerated protein turnover. In this respect our data also agree well with findings of McKee and colleagues [25], who have shown that the alkaline proteolytic activity in rat heart, an activity which is almost abolished after treatment of the rats with compound 48/80, a mast cell degranulating agent, contributes very little to protein degradation as measured by free amino acid release in the perfused rat heart.

Finally, a different response of heart muscle and skeletal muscle to diabetes mellitus is underscored by the different changes of total aminopeptidase activity in the two tissues. While this activity is significantly lower in hearts of diabetic versus control animals, studies in this laboratory have shown [13] that there is a pronounced increase in aminopeptidase activity in skeletal muscle of rats after induction of diabetes mellitus.

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